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Review

The future of antiviral immunotoxins

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ABSTRACT

There is a constant need for new therapeutic interventions in a wide range of infectious diseases. Over the past few years, the immunotoxins have entered the stage as promising antiviral treatments. Immunotoxins have been extensively explored in cancer treatment and have achieved FDA approval in several cases. Indeed, the design of new anticancer immunotoxins is a rapidly developing field. However, at present, several immunotoxins have been developed targeting a variety of different viruses with high specificity and efficacy. Rather than blocking a viral or cellular pathway needed for virus replication and dissemination, immunotoxins exert their effect by killing and eradicating the pool of infected cells. By targeting a virus-encoded target molecule, it is possible to obtain superior selectivity and drastically limit the side effects, which is an immunotoxin-related challenge that has hindered the success of immunotoxins in cancer treatment. Therefore, it seems beneficial to use immunotoxins for the treatment of virus infections. One recent example showed that targeting of virus-encoded 7 transmembrane (7TM) receptors by immunotoxins could be a future strategy for designing ultraspecific antiviral treatment, ensuring efficient internalization and hence efficient eradication of the pool of infected cells, both in vitro and in vivo. In this review, we provide an overview of the mechanisms of action of immunotoxins and highlight the advantages of immunotoxins as future anti-viral therapies. *J. Leukoc. Biol.* 99: 000–000; 2016.

Introduction

Despite modern prevention and treatment strategies, viral infections and subsequent diseases remain a major health concern with devastating consequences associated with morbidity, mortality, and burdensome economic consequences. Currently, licensed drugs for the treatment of viral infections have several drawbacks, including toxicity and emergence of drug resistance. Hence, new and improved antiviral therapies with novel modes of action are urgently needed, and use of antiviral immunotoxins as "smart bombs" provide 1 such option.

Immunotoxins are fusion molecules that consist of a targeting molecule conjugated to a toxin molecule. The targeting molecule can be either antibody based, such as a mAb and a genetically engineered single-/double-chain antibody fragment or a receptor ligand, such as a growth factor or cytokine that targets specific cell surface receptors (**Fig. 1**) [1, 2]. The targeting molecule can be fused to a range of toxins, such as bacterial, plant, or fungal toxins, or to human apoptotic proteins. Although the toxin ensures efficient killing of the diseased or infected target cell, the targeting moiety ensures selectivity (i.e., that the killing moiety is directed to the diseased cells only). The selectivity must be accompanied by efficient internalization of the immunotoxin. Thus, selection of the appropriate immunotoxin target not only relies on the target expression profile, but must also take target internalization into account [3]. Furthermore, immunotoxins are highly immunogenic molecules and may rapidly elicit an immune response when administered to humans. Improvement in design of the immunotoxins is therefore warranted (e.g., identification and silencing of human T cell epitopes [4]) to overcome resistance and to achieve low immunogenicity for repeated treatment cycles.

Mostly, but not exclusively, immunotoxins are purpose-built to kill cancer cells. Thus, the identification of numerous unique cancer targets in recent years has led to the development of various malignancy-directed immunotoxins, with successful approval by the FDA (**Table 1**). The early immunotoxin denileukin diftitox (Ontak, Eisai, Inc., Woodcliff Lake, NJ, USA) with the targeting molecule IL-2 fused to the C terminus of DT received accelerated approval for the treatment of CD-25-positive cutaneous T cell lymphoma in 1999 and received regular approval in 2008 [5–8]. A subclass of immunotoxins, known as immunoconjugates, contains an antibody for specific antigen targeting. The antibody is coupled to a variety of effector molecules by cleavable or uncleavable linkers that ensure optimal delivery of the effector molecule into the cell (**Fig. 1**). Immunoconjugates can be both highly specific and effective and with minimal toxicity if they are optimally designed. Therefore, certain immunoconjugates are considered among the most

Abbreviations: 7TM = 7 transmembrane, ACD = antibody drug conjugate, BLT = bone marrow-liver-thymus, CD = Castleman's disease, DT = diphtheria toxin, Env = envelope, FDA = U.S. Food and Drug Administration, FTP = fusion toxin protein, gp = glycoprotein, GPCR = G protein-coupled receptor, HCMV = human CMV, HCV = hepatitis C virus, KSHV = Kaposi sarcoma-associated herpes virus, MCD = multicentric Castleman's disease,

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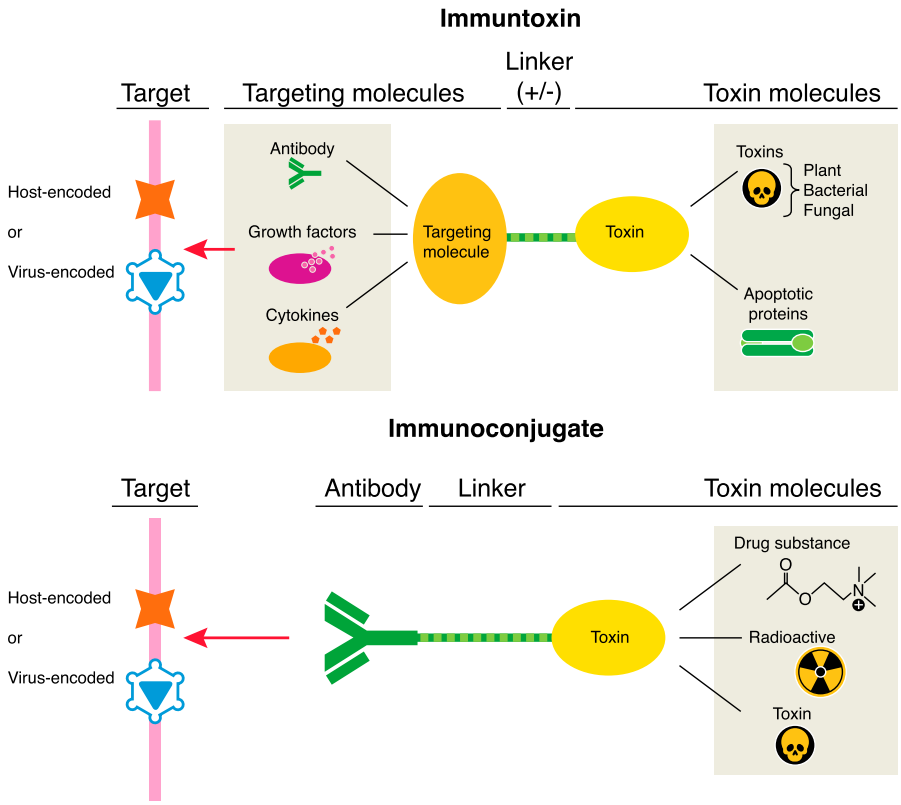

Figure 1. Structure of immunotoxins and immunoconjugates. Immunotoxins are composed of a target molecule, that can be in the form of an antibody, a growth factor, or a cytokine that directs the immunotoxin to its specific molecular target, which is cell or virus derived. The target molecule and the therapeutic can be directly fused to each other or connected by a linker molecule. The therapeutic consists of a toxin molecule derived from plants, bacteria, or fungi or an apoptotic protein that will kill the targeted cell when internalized. Immunoconjugates always have an antibody as the target molecule, which is specific to cell- or virus-derived targets. The target molecule is combined to the therapeutic by a linker, and the therapeutic is a drug substance, a radio ligand, or a toxin.

promising anticancer therapies in the clinic [9]. There are 3 classes of immunoconjugates, based on the mechanism of action of the therapeutic agent conjugated to the antibody. The first class includes a pharmacological drug-like substance; members of this class are also known as ADCs. The second class consists of radionucleotides and the third class, of catalytic toxins (Fig. 1) [9]. The 2 most well-described immunoconjugates to date belong to class 1: brentuximab vedotin (targeting CD30) and trastuzumab emtansine (targeting HER2), approved by the FDA for the treatment of certain lymphomas and breast cancer, respectively [10, 11]. Radionucleotide immunoconjugates (class 2) (e.g., ibritumomab tiuxetan and iodine tositumomab) have shown clinical efficacy in the treatment of hematologic malignancies, but have failed in solid-tumor management [12–14]. Several immunoconjugates that involve a catalytic toxin (class 3) show promising results and are under clinical evaluation (phase I–III trials) and preclinical trials [9, 15]. Table 1 provides an overview of immunotoxins for treatment of cancers. Given the impressive progress in anticancer immunotoxin development, in particular regarding efficacy and safety, there is great potential of this technique for other clinical indications, such as infectious diseases, where pathogen-encoded targets provide superior specificity as compared to up-regulated endogenous target molecules in cancers. In the this review we focus on immunotoxins for the treatment of virus infections. At present, all antiviral immunotoxins are antibody based, yet 1 exemption recently entered the stage by presentation of the first antiviral immunotoxin targeting a virus-encoded 7TM GPCR denoted US28—not by an antibody, but by refinement of a chemotactic cytokine (chemokine) for optimal binding to US28 [16]. We discuss existing antiviral immunotoxins, novel viral targets for immunotoxins with main focus on virus-encoded 7TM GPCRs, and perspectives on the future of immunotoxin-based antiviral therapy.

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MCMV = murine CMV, PCV = Pichinde virus, pAb = polyclonal antibody, PE = *Pseudomonas* exotoxin A, PEL = primary effusion lymphoma, PTLD = posttransplant lymphoproliferative disease, vGPCR = viral GPCR, VHH = heavy-chain-only antibody, WT = wild-type

IMMUNOTOXINS AS NOVEL ANTIVIRAL THERAPEUTICS

Today's antiviral therapies intervene in the infection cycle of the virus by primarily targeting virus entry, intracellular virus replication, virus particle formation, and cell exit or by modulating cellular immune defense systems [17]. Immunotoxins as antiviral therapeutics are relatively underexploited, but are potentially useful, as these molecules can interfere with virus replication at the same time as killing virus-infected cells [16, 18]. The greatest advantage, in comparison with anticancer immunotoxins, is that the target molecule often is encoded by the virus, thereby preventing side effects encountered by killing of cells expressing low levels of the targeted human molecule (**Table 2**). In addition, interference with the protein synthesis within infected eukaryotic cells blocks virus dissemination and may eliminate the reservoirs in latently infected cells from which the virus reactivates [19].

TABLE 1. Characteristics and developmental state of immunotoxins used for cancer treatment. The immunotoxins are listed in alphabetic order. Molecular targets of the immunotoxins are shown in bold. Immunotoxins described in the text are shown on grey background.

Immunotoxin	Targeting moiety: molecular target	Toxin moiety	Type of cancer	Developmental stage	References
260F9 Mab-rRA	Anti-M_r 55,000 murine mAb (260F9): **M_r 55,000 antigen**	RicA	Breast cancer	Terminated in phase I (1989)	[86, 87]
454A12-rRA	Anti-human TFR mAb (454A12): **TFR**	RicA	Ventricular cerebrospinal fluid cancer	Phase I	[88, 89]
A-dmDT390-bisFv	Anti-CD3 with addition of an extra sFv domain: **CD3**	DT	CTCL	Phase I	[90, 91]
Ado-trastuzumab emtansine (Kadcyla)	Anti-HER2 mAb (trastuzumab): **ErbB2**	DM1[a]	Breast cancer	FDA approved (22.02.2013)	[11, 92–100]
Anti-B4-bRicin	Anti-CD19 mAb: **CD19**	Blocked Ricin	B-cell non–Hodgkin lymphoma	Terminated in phase III (2011)	[101–106]
Anti-MY9-bRicin	Anti-CD33 mAb (MY9): **CD33**	RicB	AML	Terminated in phase I (1998)	[107];
B43-PAP	Anti-CD19 mAb (B43): **CD19**	PAP	ALL	Phase I	[108, 109]
Ber-H2-Sap6[c]	Anti-CD30 mAb (Ber-H2): **CD30**	Saporin 6	Hodgkin disease	Phase I	[110–112]
BL22 (RFB4(dsFv) PE38 or CAT-3888)	Disulfide-linked V_H and V_L chains of Anti-CD22 Fv fragment (RFB4): **CD22**	PE-38	B-cell malignancy	Phase II trials completed, superseded by moxetumomab pasudotox	[113–117]
Brentuximab vedotin (Adcetris)	Mouse-human chimeric IgG1 anti-CD30 mAb: **CD30**	MMAE[a]	Hodgkin lymphoma; anaplastic large cell lymphoma	Accelerated FDA approval (10-19-2011)	[10, 118–123]
Cintredekin besudotox	IL-13: **IL-13Rα2**	PE truncated form, PE38QQR	Malignant glioma	Terminated in phase III (2010)	[124–127]
DA7	Mouse anti-CD7 human mAb: **CD7**	dgA	T-cell non-Hodgkin lymphoma	Phase I	[128, 129]
DAB_{486}IL-2[c]	IL2: **CD25**	DAB_{486}	Hematologic cancer	Phase I/II	[130–134]
Denileukin diftitox (ONTAK)[c]	IL2: **CD25**	DAB_{389}	CTCL	FDA approved	[7, 135–141]
DT390-GMCSF and DT388-GM-CSF	GM-CSF: **GM-CSFR**	DT390	AML	Phase I	[142–144].
DT388-IL3	IL-3: **IL-3R**	DT388	AML	Phase I/II[b]	[145, 146]
erb-38	Disulfide stabilized Fv portion of mAb e23 dsFv: **ErbB2**	PE38	Breast cancer	Phase I	[147–149]
Gemtuzumab ozogamicin (Mylotarg)	Anti CD33 mAb (hP67.6): **CD33**	Calicheamicin	AML	FDA approved (05-17-2000) but later withdrawn (10-15-2010)	[150][b], [151–154]
H65-RTA	Anti-CD5 mAb (H65): **CD5**	RicA	CTCL	Phase I/II	[155, 156]
HD37-dgA	Anti-CD19 mAb (HD37): **CD19**	dgA	B-non Hodgkin lymphoma	Phase I	[157, 158]
HUM-195/rGEL	Anti-CD33 humanized mAb (M195): **CD33**	rGel	Refractory myeloid leukemias	Phase I	[159–161]

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TABLE 1. (*continued*)

Immunotoxin	Targeting moiety: molecular target	Toxin moiety	Type of cancer	Developmental stage	References
IL4-PE	Circularly permuted IL-4	PE38KDEL	Malignant glioma	Phase I /II	[162–165]
Ki-4.dgA	Anti-CD30 mAb: **CD30**	dgA	Hodgkin's disease	Phase I/II	[166, 167]
B3(LysPE40) and LMB-1 (B3(LysPE38))	Anti-Le^Y mAb (B3): **Lewis Y**	LysPE-40 and LysPE-38	Breast cancer and colon cancer	Phase II	[168–170]
Anti-TAC(Fv)PE40 and LMB-2 (anti- TAC (scFv)PE38)	Anti-CD25 Fv portion (anti-tac): **CD25**	PE40	$CD25^+$ T- and B- cell malignancies	Phase I, recruiting for a phase II study	[171–173].
LMB-7 (B3(Fv)–PE38)	Anti-Le^Y mAb single chain Fv fragment [B3 (Fv)]:**Lewis Y**	PE-38KDEL	Neoplastic meningitis	Terminated in phase I (2000)[b]	[174]
LMB-9 (B3(dsFv)–PE38)	Stable disulphide version of B3(Fv) [B3(dsFv)]: **Lewis Y**	PE-38	Bladder, lung, breast, GI-tract and pancreatic cancers	Terminated in phase I (2003)[b]	[175]
Moxetumomab pasudotox	RFB4 with point mutations Ser100Thr, Ser100aHis and Tyr100bTrp in heavy-chain CDR3: **CD22**	PE-38	B cell malignancies	Phase I, recruiting for a phase II study phase I[b]	[176, 177]
MR1-1	Anti-EGFRvIII mAb scFv fragment: **EGFR vIII**	PE38KDEL	Glioblastoma multiforme	Phase I[b]	[178]
N901-bR	Anti-CD56 mAb: **CD56**	Blocked ricin	Small cell lung cancer	Terminated in phase II (2002)	[179–181]
OVB3-PE	Anti-ovarian cancer cell mAb (OVB-3): **unknown antigen on ovarian cancer cells**	PE	Ovarian cancer	Terminated in phase I (1991)	[182, 183]
RFB4-dgA	Anti-CD22 murine mAb (RFB): **CD22**	dgA	B-non Hodgkin lymphoma,	Phase I	[184–187]
RFB4-Fab-dgA	FAb′ fragment of RFB: **CD22**	dgA	B-non Hodgkin lymphoma	Phase I	[188, 189]
RFT5-dgA[c]	Anti-CD25 Ab: **CD25**	dgA	Hodgkin's disease	Phase I/II	[190–194]
ScFv(FRP5)-ETA	N-terminal single-chain Ab fragment (scFv) specific to ErbB2: **ErbB2**	PE	Breast, head, prostate and neck cancers.	Phase I	[195, 196]
SGN-10	Linked heavy- and light-chain variable regions of murine mAb BR96 (BR96 (sFv)): **Lewis Y**	PE40	Le^Y-positive non hematological metastatic carcinomas	Phase I	[197, 198]
SS1P	Anti-mesothelin single-chain Fv Ab: **Mesothelin**	PE-38	Mesothelioma, ovarian and pancreatic cancers	Phase I, recruiting for a phase II study	[199–202]
T101-ricin A chain	Anti-65 kD GP mAb (T101): **65 kD GP**	RicA	CLL	Terminated in phase I (1988)	[203, 204]
TF-CRM107	Transferrin-C: **TFR**	DT (with mutations Leu390Phe Ser525Phe in the B chain)	Malignant glioma	Phase III study completed in 2008[b]	[205–208]

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TABLE 1. (*continued*)

Immunotoxin	Targeting moiety: molecular target	Toxin moiety	Type of cancer	Developmental stage	References
TGFα-TP40	TGF-α: **EGFR**	PE-40	Bladder cancer	Phase I	[209, 210]
TGFα-TP38	TGF-α: **EGFR**	PE-38	Malignant glioma	Phase II	[211–214]
XomaZyme-791	Murine anti-colon carcinoma mAb (791T/36): **M_r 72,000 antigens on colon carcinoma cells.**	RicA	Colon cancer	Phase I	[215, 216]
XomaZyme-MEL (or XMMME-001-RTA)	Murine anti-melanoma mAb: **melanoma antigens with a molecular weight of 220,000 and over 500,000**	RicA	Melanoma	Phase II	[217–219]

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DAB, modified diphteria toxin; DAB389, the first 389 amino acids of DT; CLL, chronic lymphocytic leukemia; CTCL, cutaneous T-cell lymphoma; dgA, deglycosylated ricin-A-chain; DM1, derivative of maytansine 1; DT, diphteria toxin; EGFR, epidermal growth factor receptor; FCRL-5, Fc receptor-like protein 5; GI, gastrointestinal; GM-CSFR, Granulocyte-macrophage colony-stimulating factor; HER, human epidermal growth factor receptor; kD, kilodalton; MC, mixed cryoglobulinemia; MMAE, Dolastatin 10 analog monomethyl auristatin e; MZ, marginal zone; PAP, pokeweed antiviral protein; PE38, genetically engineered deletion of amino acids 253-334 and 381-613 of PE; PE38KDEL, truncated form of PE where the amino acids KDEL is added in the C terminal region to increase toxicity; PE40, deletion of domain Ia of PE; rGEl, recombinant gelonin; RicA, ricin A chain; RicB, ricin B chain; TFR, transferrin receptor; VH, heavy chain domain of variable Ab region; VL, light chain domain of variable Ab region. [a]Only immunotoxins based on non-classical (not bacterial, plant or fungal derived) toxins that are FDA approved are included in the table. [b]Unpublished data. [c]Immunotoxins tested as both cancer and viral treatment.

Antibody-based immunotoxins for virus targeting

Immunotoxins have been developed against a variety of viruses, including small single-stranded RNA viruses, such as HIV, PCV, and HCV, and larger DNA viruses, such as herpesviruses. (Table 2 provides a comprehensive overview of antiviral immunotoxins). In this review we focus on the most well-investigated immunotoxins against HIV and herpesviruses.

Antibody-based immunotoxins

Antibody-based immunotoxins were assessed as a monotherapy for HIV infection soon after the identification of HIV as the causative agent of AIDS [20–23], but were found to be ineffective [24–26]. At present, immunotoxins are under consideration for incorporation into HIV eradication protocols in combination therapy [18, 24, 27]. Today's antiretroviral treatment provides a lifesaving and effective control of HIV infections [28], as it promotes dramatic reductions in viral load in blood and lymphoid tissues and is accompanied by significant recovery of $CD4^+$ T-lymphocyte counts and immune system function [29]. However, infected cell reservoirs and low-level replication of HIV persist during years of suppressive antiretroviral treatment, leading to viral rebound upon cessation of treatment [29]. Therefore, adherence to daily treatment is essential, but with the potential consequence that drug resistance of viral variants may emerge. Therefore, strategies for HIV eradication therapies have been pursued [18, 30, 31], and immunotoxins have been tested for HIV eradication in combination with an antiretroviral therapy. HIV cell entry is facilitated by the sequential interaction of the viral Env gp120 with CD4 on the surface of the cell [32]. Therefore, immunotoxins consisting of antibodies binding to gp120 linked to the truncated form of PE have been developed. The 3B3(Fv)-PE38 (hereafter, 3B3-PE) is an improved version of the CD4(178)-PE40 (hereafter, CD4-PE) immunotoxin [26, 29]. Both immunotoxins display highly potent and specific cytotoxic actions in vitro against replication of HIV in PBMCs and monocyte-derived macrophages [33, 34]. This effect is particularly noteworthy in view of the extremely low levels of surface Env expression in macrophages and the postulated role of macrophages in HIV persistence during antiretroviral treatment [34]. A thymus-liver SCID-hu mouse was used to examine the ability of both immunotoxins in combination with antiretroviral drugs to treat HIV infections in vivo. HIV tropism is confined to human tissue that is capable of supporting a productive infection of the virus. Thus, to establish HIV infection in a mouse model, SCID mice underwent implantation of human fetal thymus and liver under the mouse kidney capsule [35]. Both immunotoxins strikingly improved traditional anti-HIV treatment, but did not eradicate the infection. Recently, 3B3-PE has been tested further in a BLT humanized mouse model, which has been validated in the study of HIV persistence [18]. The process of bioengineering BLT mice results in systemic dissemination of human hematopoietic cells through the animal [36]. After effects of HIV infection on the BLT mouse human immune system were observed (e.g., $CD4^+$ T cell depletion and immune activation), antiretroviral therapy was established, and 3B3-PE was incorporated into the therapeutic regimen. 3B3-PE significantly improved the anti-HIV treatment, by profoundly depleting productively infected cells systemically, but did not eliminate the virus [18].

The tested immunotoxins can limit the size of the HIV reservoir, but cannot eradicate HIV in combination with the

TABLE 2. Characteristics and developmental state of immunotoxins used for viral treatment. The immunotoxins are listed in alphabetic order. Molecular targets of the immunotoxins are shown in bold and further in italics if the target is virally expressed. Immunotoxins described in the text are shown on grey background.

Immunotoxin	Targeting moiety: Molecular target (*viral* or human)	Toxin moiety	Type of infection	References
0.5β-RAC and 0.5β-PE	mAb 0.5β: ***gp120***	RicA/PE	HIV	[220]
3B3(Fv)-PE38	3B3 scFv Ab: ***gp120***	PE38	HIV	[221]
25-D1.16 Fab/pOV8-K^b	Heavy and light chains of TCR-like Ab 25-D1.16 Fab fragment: **pOV8 (SIINFEKL) in association with H-2K^b class I MHC (pOV8-K^b)**	PE38	Rabies virus	[222]
41.1-Ricin A	Anti-gp41 human mAb 41.1: ***aa 579–603 of gp41***	dgA	HIV	[223]
907-RAC	Anti-gp120 mAb 907: ***aa 313–324 of gp120***	RicA	HIV	[224]
924-Ricin A	Anti-gp120 mAb 924: ***aa 313–324 of gp120***	RicA	HIV	[223]
2014-PE38	Anti-KSHV K8.1A mAb (4C3): ***gpK8.1A viral glycoprotein***	PE38	$KSHV^+$ KSHV-associated cancers	[42]
Anti-CD45RO IT	Anti-CD45RO Ab: **CD45RO**	dgA	HIV	[225]
Anti-gp41-dgA (98-6-dgA + 50-69-dgA)	Anti-gp41 mAb: ***gp41***	dgA	HIV	[226]
Anti-gp160 IT	Anti-gp160 polyclonal Ab: ***gp160***	dgA	HIV	[223]
BAT123-PAP	BAT 123 mAb: ***aa 307–317 of the PND region on gp120***	PAP	HIV	[227]
Ber-H2-Sap6[c]	Anti-CD30: **CD30**	Saporin6	$CD30^+$ EBV^+ B-cell tumors	[228]
CD4(178)PE40	First 178 amino acids of CD4 [rsCD4(178)]: **gp120 binding domain of CD4**	PE40	HIV	[230, 231, 26] Terminated in phase III (1994)
CytG-gelonin	Anti-HCMV polyclonal human IgG: ***HCMV infected cells***	Gelonin	HCMV	[51]
CytG-gelonin	Anti-MCMV polyclonal IgG: ***MCMV-infected cells***	Gelonin	MCMV	[51]
DAB389CD4	rsCD4(178): **gp120 binding domain of CD4**	DAB389	HIV	[231]
DAB_{486}IL-2[c]	rIL-2: **CD25**	DAB486	HIV	[130, 232]
DAB_{389}IL-2[c]	rIL-2: **CD25**	DAB389	HIV	[135, 233]
F49A-FTP	CX_3CL1 with a single-point mutation (Phe49Ala): ***US28***	PE	$HCMV^+$ HCMV-associated glioblastoma	[16]
F58-RAC	mAb F58: ***aa 309–317 of gp120***	RicA	HIV	[234]
G3.519-PAP-S	mAb G3.519: **gp120 binding domain of CD4**	PAP-S	HIV	[227]
HB5-gelonin	Anti-C3d Mab (HB5): **EBV/C3d receptor**	Gelonin	EBV associated malignancy	[235]
HMS-dgA, C34-dgA and D5-dgA	Anti-MCMV monoclonal IgG: ***MCMV infected cells***	dgA	MCMV	[52]
HRS-gelonin	Anti-PCV mAb (HRS): ***PCV antigen***	Gelonin	PCV	[236]
PAP-anti-CD4	Anti-CD4 mAb: **gp120 binding domain of CD4** Anti-CD5 mAb: **CD5** Anti CD7 mAb: **CD7**	PAP	HIV	[237]
R33ExoA	Anti-viral surface gp D (R33) variable domains of VHH: ***Viral surface gp D***	PE	HSV-2	[19]
rCD4-dgA	rsCD4(178): **gp120 binding domain of CD4**	dgA	HIV	[238]

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TABLE 2. (*continued*)

Immunotoxin	Targeting moiety: Molecular target (*viral* or human)	Toxin moiety	Type of infection	References
RFT5-dgA[c]	Anti-CD25 mAb (RFT5): **CD25**	dgA	HIV	[239, 240]
UCHT1-DT	Anti-CD3 mAb: **CD3**	DT	HIV	[241]
YC15-PE38	Single-chain variable region fragment anti-KSHV gH mAb: ***KSHV gH***	PE38	KSHV $^{+}$ KSHV associated MCD	[41]

The immunotoxins are listed in alphabetical order. Molecular targets of the immunotoxins are shown in bold and further in italics if the target is virally expressed. Immunotoxins described in the text are shown on gray background. AML=acute myeloid leukemia; DAB=modified diphtheria toxin; DAB_{389}=the first 389 amino acids of DT; CDR=complementarity-determining regions; CLL=chronic lymphocytic leukemia; CTCL=cutaneous T-cell lymphoma; dgA=deglycosylated ricin-A-chain; DM1=derivative of maytansine 1; EGFR=epidermal growth factor receptor; FCRL-5=Fc receptor-like protein 5; gH=glycoprotein H; GI=gastrointestinal; GM-CSFR=granulocyte-macrophage colony-stimulating factor; HER=human epidermal growth factor receptor; MC=mixed cryoglobulinemia; MMAE=dolastatin 10 analog monomethyl auristatin; MZ=marginal zone; PAP=pokeweed antiviral protein; PE38=genetically engineered deletion of aa 253–334 and 381–613 of PE; PE38KDEL=truncated form of PE where the amino acids KDEL is added in the C terminal region to increase toxicity; PE40=deletion of domain Ia of PE; *PNAS*=*Proc. Natl. Acad. Sci. USA*; rGEl=recombinant gelonin; RicA=ricin A chain; RicB=ricin B chain; TFR=transferrin receptor; V_H=heavy chain domain of variable Ab region; V_L=light chain domain of variable Ab region. [a]Only immunotoxins based on nonclassic (not bacteria-, plant-, or fungus-derived) toxins that are FDA approved are included in the table. [b]Unpublished data. [c]Immunotoxins tested as both cancer and viral treatments.

antiretroviral therapy. However, based on the results, alternative immunotoxins with different effector molecules and targeting moieties (Table 2) could be successful components of an effective HIV eradication therapy.

Antibody-based immunotoxins targeting herpesviruses

Antibody-based immunotoxins targeting herpesviruses have shown promising results in several cases, as discussed in detail below.

Antibody-based immunotoxins against KSHV. KSHV (also known as HHV-8) is an oncogenic virus that has been characterized as the etiologic agent in the onset and development of KS, PEL, and MCD [37, 38]. Like other herpesviruses, KSHV possesses 2 distinct transcriptional programs: latency and lytic replication [39]. In the latent phase, viral genomes are maintained silent within the infected cells, and in the lytic phase, all lytic viral genes are expressed in a tightly regulated cascade, after which the progeny virus is assembled and released from the infected cell. Two immunotoxins have been developed for selective killing of lytically infected cells: YC15-PE38 and 2014-PE38 [40, 41]. They both consist of a single chain Fv mAb targeting KSHV lytic gps linked to the truncated form of PE (PE38). The targeted gps are expressed on KSHV particles to facilitate virus entry, and consequently, the spread of the infection in the human host. YC15-PE38 binds to the KSHV gpH [40] and the antibody fragment of 2014-PE38 is against gpK8.1A [42]. Both immunotoxins have been shown to inhibit the production of infectious KSHV particles and specifically to kill KSHV-infected cells in a dose-dependent fashion in vitro. In particular, 2014-PE38 was observed to be efficient, even under conditions of very low gpK8.1A expression, and has therefore been discussed as a potential treatment for MCD, as this cancer is mostly associated with the lytic phase of the viral life cycle, when gpK.8.1A is expressed [41].

Antibody-based immunotoxin against HSV-2. HSV-2 is one of the most prevalent sexually transmitted infections [43]. In addition to recurrent genital ulcers, HSV-2 causes neonatal herpes and is associated with a 3-fold increased risk for HIV acquisition [44]. Structurally altered antibodies produced by the camelid family are currently exploited for the treatment of HSV-2. Members of this family (camels, llamas, and alpacas) can produce antibodies that lack light chain and CH1 domain, also denoted VHHs [45]. VHHs demonstrate the same antigen-binding capability as full-length antibodies and are purified as monomeric domains. They demonstrate remarkable stability in a wide range of denaturing, temperature, and pH conditions. These properties of VHH have been exploited in the development of an immunotoxin for intravaginal use in the treatment of HSV-2 [46].

Using a phage display library constructed from a llama immunized with a recombinant HSV-2-encoded gpD, a single-domain antibody VHH, R33, was identified with specific binding to the viral cell surface gpD. R33 did not demonstrate any HSV-2 neutralization. However, fusion of R33 to the cytotoxic domain of PE yielded an immunotoxin (R33ExoA) which, unlike existing antivirals targeting HSV-2, was highly efficient in killing virus-infected cells in vitro [46]. R33ExoA is the first immunotoxin designed for targeting a virus at a mucosal site of infection, but to be considered for anti-HSV-2 therapy, its efficacy under exceptional conditions in vivo, such as low vaginal pH, has to be proven.

Antibody-based immunotoxins against HCMV. HMCV can cause severe diseases in immunocompromised persons and establishes life-long infection in the human body [47]. Because the virus is highly species specific, it is not easily studied in animal models (except humanized mouse models). Instead, its murine counterpart MCMV is often used as a surrogate model for the study of virus replication and pathogenesis in vivo [48, 49]. Immunotoxins specific for cells infected with HCMV and MCMV have been constructed by linking polyclonal anti-HCMV and -MCMV IgG to gelonin, a type I ribosome- inactivating protein. Both immunotoxins failed to obtain sufficient anti-HCMV/anti-MCMV activities in vitro [50], indicating that other immunotoxins with improved antiviral activities are needed for in vivo studies. Examples of these are mAbs and pAbs conjugated to a deglycosylated ricin A chain for MCMV targeting. These

immunotoxins were more efficient than the gelonin-based anti-MCMV immunotoxin and were therefore evaluated in vivo in MCMV-infected SCID mice [51]. Unfortunately, they were both inadequate in their effects on survival [52] and their overall effect was weak in comparison to that of ganciclovir in mice. Further work is needed to determine whether more potent and efficient antibody-based immunotoxins against CMV can be found.

CHEMOKINE-BASED IMMUNOTOXINS FOR HCMV TARGETING VIA US28

The first chemokine-based immunotoxin targeting a viral GPCR has been reported recently [16]. The targeting molecule was not antibody-based, but instead was created by a series of modifications in the cognate chemokine-ligand for the HCMV-expressed chemokine receptor US28. GPCRs constitute the biggest protein family in the human genome and are targets for ~50% of all currently marketed drugs [53]. Based on their 7TM α-helices, they are also known as 7TM receptors. The chemokine receptors and ligands constitute important parts of a very complex system that controls leukocyte movements during homeostasis and inflammation. Herpesvirus genomes have evolved an extraordinary repertoire of tools designed to ensure successful infectivity and propagation. A large fraction of these tools involves virus-encoded 7TM receptors and ligands, most of which have high genetic and functional homology to either chemokines or chemokine receptors. Thus, HCMV devotes a significant part of its genome to immune modulatory gene homologs, as the virus-encoded GPCRs UL33, UL78, US27, and US28, with US28 being a functional chemokine receptor [54]. Several pharmacological and cellular properties of US28 suggest that this vGPCR would be suitable for targeting of HCMV in an immunotoxin-based strategy. First, although US28 binds a broad spectrum of chemokines as part of its proposed immune evasive function as a chemokine scavenger, it shows high selectivity and enhanced binding for the CX_3C chemokine CX_3CL1 [55]. Because CX_3CL1 binds only 1 human chemokine receptor, CX_3CR1, the potential of unwanted off-target effects of a CX_3CL1-based immunotoxin strategy is decreased. Second, CX_3CL1 consists of a chemokine domain, a mucin stalk, and a transmembrane domain. These structural characteristics suggest that this chemokine can sustain high-affinity binding to US28 when the C-terminally attached mucin-like stalk is replaced by another protein (e.g., the cytotoxic domains of PE). Third, detailed studies indicate that most of the US28 receptors localize away from the cell surface in endosomes [56, 57]. This distribution results from rapid, constitutive, and ligand-independent receptor internalization [58], a feature well-suited for efficient intracellular delivery of immunotoxins. Based on the molecular characteristics of US28 and its defined ligand profile, an immunotoxin was designed consisting of the chemokine domain CX_3CL1 and the translocation and cytotoxicity domains of PE (**Fig. 2**). It was given the name CX_3CL1-FTP to highlight that the target moiety was based on a chemokine, not an antibody [16]. CX_3CL1-FTP binds to US28 with higher affinity, kills US28-expressing cells with higher potency, and more efficiently controls virus replication and release of virus particles than does ganciclovir. Moreover, CX_3CL1-FTP is capable of controlling virus replication of a ganciclovir-resistant HCMV strain—a highly important property for treatment of patients who have an increasing clinical challenge of infection with ganciclovir-resistant HCMV strains. As CX_3CL1-FTP also killed cells expressing the human chemokine receptor CX_3CR1 (albeit much less efficiently), a rational design strategy was applied to further enhance the selectivity of the immunotoxin molecule toward US28 [16]. A single-point mutation (Phe^{49} to Ala) in CX_3CL1 retained high-affinity binding to US28 and reduced affinity to CX_3CR1, providing a favorable selectivity profile toward US28. The selectivity-optimized immunotoxin F49A-FTP exhibited far superior control of HCMV infection in vivo in the SCID-hu mice model compared with ganciclovir [16, 59]. The efficacy, combined with the rational design of a selective chemokine as the target molecule, demonstrates the high therapeutic potential of this drug candidate. Many questions clearly remain, including the capacity for development of F49A-FTP-resistance in vivo and toxicity and the immunogenicity associated with F49A-FTP.

7TM receptors as potential antiviral immunotoxin targets

As evident from the presentation of F49A-FTP, the immunotoxin drug candidate for the treatment of HCMV, rationally designed immunotoxins targeting virus-expressed receptors may provide promising drug targets, not only for anti-HCMV therapy, but also other virus-encoded 7TM receptors [60–63].

The EBV-encoded BILF1 receptor. EBV encodes 1 constitutively active tumorigenic receptor, EBV-BILF1 [61, 62, 64]. It has been suggested that this 7TM receptor is involved in the pathogenesis of EBV-associated malignancies, presumably in a signaling-dependent manner. The molecular properties of EBV-BILF1 indicate that this receptor is a promising drug target and is suitable for immunotoxin targeting. Cell surface expression of EBV-BILF1 can be detected during the lytic phase of infection, and even low receptor expression levels were detectable in latency [65]—an important feature for targeting EBV-associated cancers as only a few viral gene products are expressed. Moreover the receptor is constitutively internalized into the cell [66], which is important for efficient toxin delivery into cancer cells. However, as EBV-BILF1 is an orphan receptor, a ligand has to be identified for the immunotoxin design.

The KSHV-encoded chemokine receptor ORF74. This broad-spectrum chemokine receptor seems highly suitable for immunotoxin targeting. In itself, ORF74 can induce the onset of Kaposi's sarcoma–like lesions through the activation of a complex network of signaling pathways that involve the autocrine and paracrine activation of proliferative, proinflammatory, and angiogenic pathways. Targeting ORF74-mediated signaling by inhibiting individual pathways has been shown to have insufficient therapeutic potential to cure several cancers [67]. In contrast, using immunotoxins designed to target KSHV-infected cells through ORF74 seems a valid approach to efficiently kill KSHV-infected cells; hence, the abrogation of oncogenic signaling events. ORF74 has a defined chemokine ligand profile [68–70] and is internalized in response to human CXCL1 and -8 [71]. Thus, a rational chemokine-based immunotoxin strategy in homology to the strategy outlined above for US28 and CX_3CL1 could be applied.

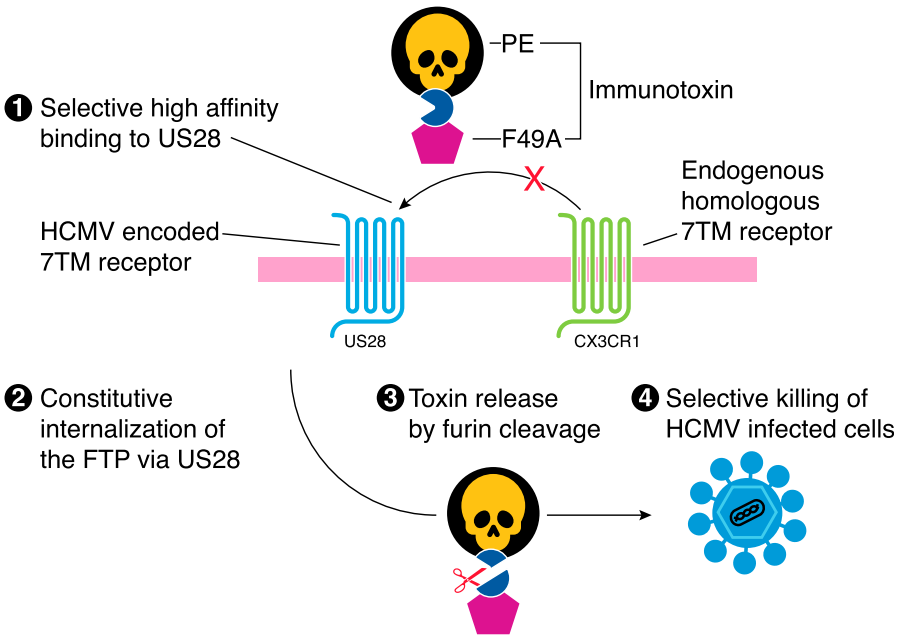

Figure 2. Selective killing of HCMV infected cells by F49A-FTP. The FTP consisting of the CX_3CL1 variant F49A and the cytotoxic domains of PE binds selectively to US28 (*1*), and the internalization of the FTP is triggered by internalization of the bound CX_3CL1 variant to US28 (*2*). The release of the CX_3CL1 variant is achieved by proteolytic cleavage (*3*), and the protein synthesis is inhibited by PE, leading to the killing of the infected cell (*4*).

In addition to the viral 7TM receptors, several viruses exploit host-encoded receptors by different means: *1*) by up-regulating them in the same manner that EBV up-regulates EBI2 [72], *2*) by using them as cell entry cofactors [73–76], or *3*) by encoding ligands (agonists and antagonists) for endogenous receptors, as vMIP1–3 is encoded for KSHV [77–79]. In theory, immunotoxins could also target these 3 methods. For the up-regulated receptors, such as EBI2 (also known as GPR183), it has been suggested that the up-regulation improves virus replication, and that it could be important for antiviral immune defense [80, 81]. The role of EBI2 in the EBV life cycle is still uncertain [81]. If EBV benefits from high EBI2 expression, an immunotoxin targeting EBI2 could provide an efficient and specific way to inhibit EBV-associated diseases. A possible drawback of this approach would be cytotoxic side effects in all EBI2-expressing cells (B cells, T cells, macrophages, dendritic cells, and many others) [82]—effects that could be reduced by lowering the dose. However the reduction, in turn, could lead to inefficient immunotoxin targeting [83, 84].

FUTURE DIRECTIONS AND LIMITATIONS OF ANTIVIRAL IMMUNOTOXINS

For future antiviral immunotoxins to be successful, they must be efficient, with low toxicity. This efficiency can be obtained by using the following criteria: *1*) The target of the immunotoxins must be highly disease-specific to limit side effects. Immunotoxins targeting the viral genome or virus-encoded proteins expressed on the surface of virions and infected cells are efficient targets, as these molecules are not normally expressed on human cells. *2*) The target must be expressed in both the latent and the lytic phase of the virus cycle, to completely eradicate the virus infection. Indeed, although effective killing of lytically infected cells is not enough to eradicate viruses that also have a latent stage, it is sufficient in most cases to treat acute infection. *3*) The target expressed on the surface of infected cells must have a rapid internalizing capability for the immunotoxin to be efficiently delivered to the intracellular environment. *4*) The immunotoxin must bypass the host's immune system as generation of neutralizing antibodies to the immunotoxin prevents continued treatment and retreatment. It is especially the toxin moiety of the immunotoxin that can be immunogenic, as this is often derived from bacteria or plant (nonhuman) sources. To circumvent this problem, patients could be treated with immunosuppressive drugs to prevent or delay the production of neutralizing antibodies [85].

CONCLUSION

Current development of antiviral immunotoxins is an emerging field with constantly improved and innovative methods for successful application. Research has developed combination therapies with immunotoxins that are beneficial in viral treatment, which further highlights the promise of successful application of antiviral immunotoxins.

AUTHORSHIP

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DISCLOSURE

The authors declare no conflicts of interest.

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